Growth and Ectomycorrhizal Development of Loblolly Pine Seedlings in Fumigated Soil Infested with the Fungal Symbiont Pisolithus tinctorius

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Abstract. Vegetative mycelium in a vermiculite-peat moss-nutrient medium and basidiospores of *Pisolithus tinctorius* were used to infest fumigated soil and synthesize ectomycorrhizae on over 4000 *Pinus taeda* seedlings in 36 nursery microplots. Soil mulches provided four soil temperature regimes with diurnal fluctuations between 19°C and 47°C. In all regimes, mycelial inoculum of *P. tinctorius* completely colonized roots of loblolly pine seedlings during an 8 mo growth period, formed ectomycorrhizae on approximately 92 percent of short roots, and stimulated twice as much dry matter production as in seedlings ectomycorrhizal with naturally occurring fungi, including *Thelephora terrestris*. When basidiospores of *P. tinctorius* were added to soil around 2-mo-old seedlings already ectomycorrhizal with naturally occurring fungi, approximately half the ectomycorrhizae after 8 mo were formed by *P. tinctorius*. Basidiocarps of *P. tinctorius* were produced around certain microplots. The following year, *P. tinctorius* from overwintered inoculum in the microplots formed ectomycorrhizae on loblolly pine seedlings when soil was mixed at ratios as great as 1:10 with recently fumigated soil. Vegetative mycelial and basidiospore inocula of *P. tinctorius* can be used to artificially infest fumigated soil to tailor pine seedlings with this highly beneficial ectomycorrhizal association. ***Forest Sci. 21:245–254.***

Additional key words. *Pinus taeda*, *Cronartium fusiforme*, foliar and soil chemical analyses, reforestation and afforestation practices.

THE SIGNIFICANCE of ectomycorrhizal fungi for normal growth and development of pines throughout the world is well documented. Some species of these symbiotic fungi are more beneficial to pines under certain soil conditions than others. Mikola (1973) reviewed this aspect of ectomycorrhizae. For example, loblolly pine (*Pinus taeda* L.) seedlings with ectomycorrhizae formed by *Pisolithus tinctorius* (Pers.) Coker and Couch survived and grew significantly better at 40°C than seedlings ectomycorrhizal with *Thelephora terrestris* Ehrh. ex Fr. or nonmycorrhizal seedlings (Marx and Bryan 1971). *P. tinctorius* is capable of forming ectomycorrhizae and stimulating growth of loblolly pine seedlings at soil temperatures between 14° and 34°C, while *T. terrestris* formed ectomycorrhizae at soil temperatures of 14° to 29°C but stimulated seedling growth much less than *P. tinctorius* at 29°C (Marx and coworkers 1970a).

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The ability of *P. tinctorius* to form ectomycorrhizae and stimulate growth of pine seedlings at high soil temperatures probably accounts, at least partially, for its widespread distribution on pines and birches growing on strip-mined coal wastes (Hacskaylo 1972; Hile and Hennen 1969; Schramm 1966). We observed large numbers of basidiocarps of *P. tinctorius* (as many as 3000 basidiocarps per hectare) under young pines on all coal spoils examined in the northeastern and Appalachian regions of the United States, and on kaolin spoils in Georgia. This fungus is also a component of the mixed ectomycorrhizal fungal populations in pine forests adjacent to the spoils, which undoubtedly accounts for its initial establishment by way of basidiospores from the forest onto the spoils. Few other ectomycorrhizal fungi exist on roots of young trees on these spoils. The ecological adaptation of *P. tinctorius* is perhaps mediated not only through its ability to tolerate high soil temperatures, but also by its tolerance of extreme soil pH and high levels of sulfur, aluminum, and manganese (Schramm 1966).

Considering geographic distribution (Mikola 1969) and *Pinus* host range (Marx and Bryan 1970), *P. tinctorius* is potentially useful for improving survival and growth of pine seedlings on adverse afforestation areas. However, *P. tinctorius* rarely occurs naturally on roots of seedlings from forest nurseries. *T. terrestris* is one of the major ectomycorrhizal fungi on nursery-grown seedlings (Marx and coworkers 1970b), especially in the eastern United States. The lack of ecological adaptability of *T. terrestris* to adverse soil conditions may explain why so many nursery-grown seedlings planted on adverse sites die or remain stunted for many years. Our observations indicate that seedlings which survive become heavily ectomycorrhizal with *P. tinctorius* which replaces *T. terrestris* on the roots.

In order to test *P. tinctorius* on pines on a broad scale, practical techniques for artificial infestation of nursery soil must be developed. It may be necessary to raise soil temperatures to discourage naturally occurring ectomycorrhizal fungi and to encourage root colonization by *Pisolithus* from introduced inocula. The three purposes of the present study were: (1) to test the feasibility of introducing vegetative mycelium and basidiospores of *P. tinctorius* into fumigated soil as inocula for ectomycorrhizal development; (2) to determine the effects of soil temperature, as altered by different soil mulches, on ability of *P. tinctorius* to form ectomycorrhizae on loblolly pine seedlings; and (3) to determine the overwintering ability of *P. tinctorius* in soil in the absence of host plants.

Materials and Methods

Inoculum Preparation. *P. tinctoris* (isolate 55) was grown at 25°C for 3 wk in plates of modified Melin-Norkrans (MMN) agar medium containing glucose instead of sucrose (Marx 1969). Mycelium agar discs (88 mm dia) were used to initiate mass cultures for inoculum. The inoculum containers were 2-liter jars containing a mixture of 1450 cc of grade 2 vermiculite, 50 cc of finely divided peat moss, and 750 cc of liquid MMN medium with glucose. The lid of each jar was modified with a 1 mm thick, heat resistant, plastic disc through which a pyrex glass tube (6 × 2 cm) filled with cotton was inserted. The disc and tube joint was attached with a heat resistant, silicone rubber compound (RTV-102, General Electric Company, Waterford, N.Y.). The disc with tube was then attached to the metal threaded lid with the same adhesive. The containers were autoclaved 30 min and each inoculated with eight mycelium-agar discs of *P. tinctorius*. The lids were then wrapped with parafilm to reduce microbial contamination. After 15 wk at room temperature, the vermiculite particles were permeated with mycelium of *P. tinctorius* as determined by microscopic examination.

To prepare mass inoculum for infestation of soil, mycelium was removed from the jars, passed through a 5 mm mesh screen, and held with two layers of cheesecloth while being leached with approximately 8 liters of cool running tap water.

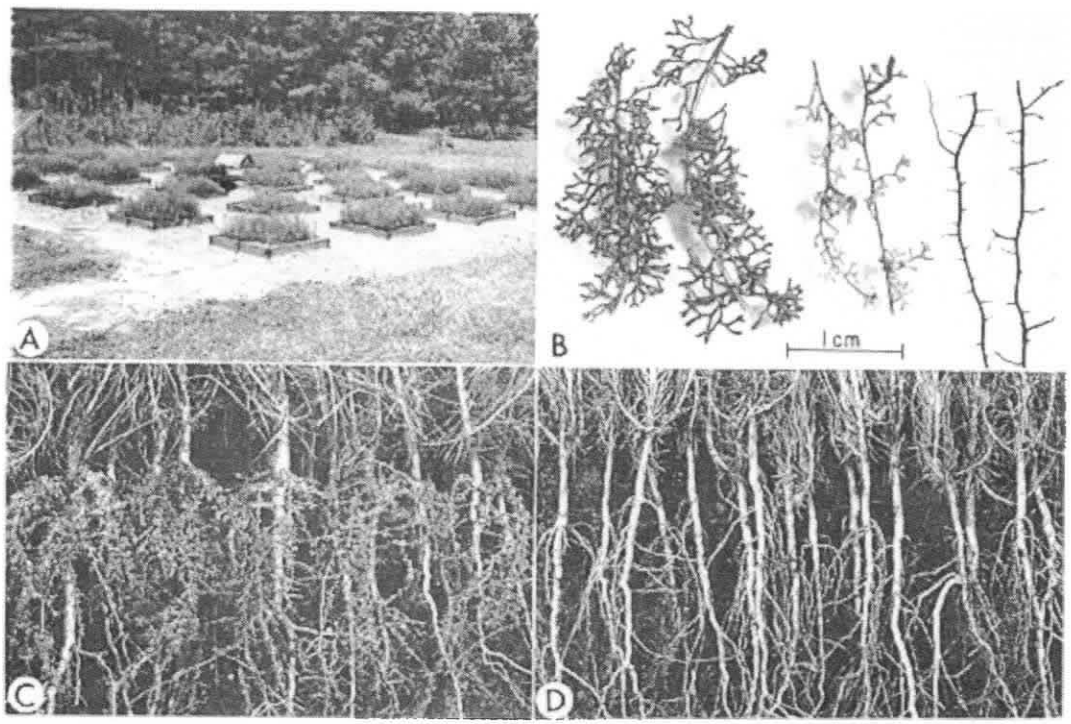

FIGURE 1. *Infestation of fumigated soil with* Pisolithus tinctorius *and ectomycorrhizae of loblolly pine.* A, *wood-frame microplot layout;* B, *ectomycorrhizae formed by* P. tinctorius (*at left*), *unidentified white fungus* (*center*), *and* Thelephora terrestris (*at right*); C, *roots of seedlings with abundant ectomycorrhizae formed by* P. tinctorius; *and* D, *roots of seedlings that became ectomycorrhizal with naturally occurring fungi in noninoculated plots.*

Excess water was removed by squeezing. The leached inoculum was placed in plastic bags and refrigerated at 5°C for 24 hr until used. Preliminary tests had indicated that nonassimilated nutrients must be leached from the inoculum. When nonleached inoculum was added to fumigated soil, saprophytic microorganisms which naturally colonize fumigated soil reproduced rapidly and significantly increased damping-off of seed, as well as reduced inoculum potential of the fungal symbiont.

Basidiospores of *P. tinctorius* were obtained from mature basidiocarps (those with exposed, dry spores) collected in June 1972 from around 3-yr-old lobolly pines growing on a strip-mined coal spoil in northeast Alabama. Over 250 mature basidiocarps 6 to 12 cm in diameter were collected from a 480 m^2 area. The basidiospores were removed by crushing basidiocarps on a 0.84 mm mesh screen in a closed plastic bag. Basidiospores passed through the screen; remnants of basidiocarps retained on the screen were discarded. Using this technique, we collected well over 1 kg (fresh weight) of basidiospores in less than 3 hr. The spores were stored dry in small plastic bags or amber glass bottles in darkness at 5°C for 10 days before use. Quantities of spores were determined with a haemocytometer; there were approximately 1.1 million basidiospores per mg.

Soil Mixing, Fumigation, and Infestation. Thirty-six microplots (0.9 m × 0.9 m × 25 cm deep) were constructed of redwood 2 cm thick. The microplots were placed 1.3 m apart on level, recently tilled soil. Soil was mounded 15 cm deep around the outside of each microplot (Fig. 1A). The entire area of microplots was covered with 4 mil polyethylene and fumigated with 450

g methyl bromide (Dowfume MC-2, Dow Chemical Co., Midland, Michigan 48640) per 12 m^2 of soil surface. The plastic cover was removed after 2 days.

The soil mixture in the microplots was a 3:1:1 (v/v/v) mixture of screened (1 cm mesh) sandy loam forest soil, 0.5 cm size pine bark, and sand. The mixture was prepared in an electric concrete mixer, spread 15 to 18 cm deep over polyethylene, covered with another layer of polyethylene, and fumigated with methyl bromide as described. Soil temperature during fumigation was 26° to 48°C and soil moisture was approximately 50 percent of field capacity. The cover was removed after 3 days and the soil mixture allowed to aerate for an additional 3 days before use. In mid-April, each microplot was filled to a depth of 10 cm with the fumigated soil mixture. Twelve randomly selected microplots were filled with another 10 cm layer of 1:8 (v/v) mixture of mycelial mass inoculum of *P. tinctorius* and fumigated soil. The remaining 24 microplots were filled with a similar mixture, except that the inoculum of *P. tinctorius* had been autoclaved for 1 hr. The microplots were covered with 1 cm of fumigated soil mixture and watered.

Seeds of loblolly pine (improved seed source—Eastern Tree Seed Laboratory, Macon, Georgia) were soaked in 1 percent H_2O_2 for 48 hr at 5°C to standardize seed germination. One day after soil infestation, six rows of seed were planted 12.5 cm apart in each microplot and covered with 1 cm of fumigated soil. Forty g of super phosphate (20 μg per liter soil) was broadcast evenly over the soil surface of each microplot and incorporated approximately 1 cm into the soil. Chemical analysis of the soil mixture (with pine bark removed prior to analysis) at planting was similar in all microplots (pH 5.5; extractable P, K, Ca, Mg, and Mn were 30, 52, 130, 44, and 205 μg per liter, respectively; 2.4 percent organic matter; and 0.05 percent total N). Chemical analyses were done by the Soil Testing Laboratory, Cooperative Extension Service, University of Georgia, Athens 30602. Screens (2 mm mesh) on wood frames were placed over each microplot to exclude birds and insects. During seed germination, the microplots were watered three times a week. One month after germination, the screens were removed and the seedlings were thinned to 110 to 120 per microplot. All microplots received the same volume of water for the duration of the experiment.

In late June, 12 of the 24 microplots that contained autoclaved mycelial inoculum of *P. tinctorius* were randomly selected for the basidiospore treatment. Trenches were dug 3 cm deep between seedling rows. Ten g of basidiospores (approx 11 billion spores) of *P. tinctorius* suspended in 500 cc of distilled water with 1 drop Tween 20 were poured into the trenches in each microplot. The trenches were leveled and the microplots heavily watered to wash the basidiospores into the soil. The remaining 24 microplots received the identical treatment except the basidiospores were killed with propylene oxide prior to suspension in water. This was done to standardize possible micronutrient effects of spores in the microplots.

Placement of Mulch Treatments. In early July, two wk after the installation of the basidiospore treatment, mulch treatments were applied to four groups of three microplots randomly chosen from each of the three inoculation treatments. The mulches were (1) 1 cm layer of fumigated 0.5 to 1.0 cm particles of black coal wash wastes (chemical analysis 0.4 percent total N; 0.62 percent total S; extractable P, K, Ca, Mg, Al, and Fe were 3, 40, 150, 130, 15, 350, and 175 μg per liter, respectively; pH 3.6) collected from a coal processing operation in northeast Alabama; (2) 6 mil black polyethylene (10 cm × 1 m) pinned to the soil around each row of seedlings; (3) 6 mil clear polyethylene installed as above; and (4) 4 cm layer of fumigated, uncut, loblolly pine straw. Soil thermometers were placed five and 10 cm deep in three microplots per mulch treatment. A thermograph recorded air temperatures in the center of the microplot layout. Soil temperatures were recorded several times

daily during the experiment. Experimental design was completely randomized with three ectomycorrhizal conditions and four soil mulch treatments, each replicated three times.

Seedling Growth Measurements. Height measurements of 12 randomly selected seedlings in each microplot were recorded monthly. Three root samples per microplot were removed monthly with a 2 cm diameter cork borer to a depth of 8 cm and examined for ectomycorrhizal development. Holes made by the cork borer were filled with fumigated soil.

Lindane insecticide was sprayed on seedlings in all microplots three times at monthly intervals during early seedling growth to control insects.

In mid-December, 8 mo after seeding, the dormant seedlings were carefully hand-lifted by rows and placed in buckets of water. Three seedlings from each of the four middle rows in each microplot, free of rust galls caused by *Cronartium fusiforme* Hedgc. and Hunt ex Cumm., were randomly selected and measured. Height, stem diameter at root collar, percent of short roots ectomycorrhizal, number of different morphological and color forms of ectomycorrhizae, and root and shoot dry weights (48 hr at 90°C) were recorded for each of the 12 seedlings per microplot. Seedlings with rust galls were measured for height and gall length. Needles from three rust-free seedlings and soil from each microplot were analyzed chemically.

Lateral roots with 25 to 150 representative ectomycorrhizae from each microplot were surface-sterilized in 100 μg per liter $HgCl_2$ for 2.5 min and rinsed in sterile water. The ectomycorrhizae were excised from the lateral roots and individually tubed on MMN agar medium with glucose for reisolation of the fungal symbionts (Marx and coworkers 1970b). Other representative ectomycorrhizae were fixed in weak formalin-acetic acid, dehydrated, infiltrated with paraffin, sectioned (10 μ), and stained in safranine-fast green for histological examination (Marx and Davey 1969). Representative ectomycorrhizae from each microplot were also placed in physiological saline for 5 days and then tested with a modified fluorescent antibody (FA) technique developed for *P. tinctorius* and *T. terrestris* (Schmidt and coworkers 1974). Tests with FA were performed by S. V. Krupa and E. L. Schmidt, Department of Soil Science, University of Minnesota, St. Paul.

Overwintering of Infested Soil. To determine the overwintering ability of *P. tinctorius* in the absence of a host plant, the soil of the mycelial inoculum treatment was maintained free of hosts from December 1972 through April 1973. During this period, the entire soil volume in the microplots was frozen for 2 to 3 days on several occasions. Monthly high, average, and low air temperatures and total precipitation for December 1972, and January, February, and March 1973 were 24°, 10°, and –7°C and 21 cm; 21°, 7°, and –7°C and 10.8 cm; 20°, 7°, and –8°C and 6.8 cm; and 27°, 14°, and –2°C and 25 cm, respectively. In April, the overwintered soil was mixed with recently fumigated soil from microplots of the other treatments at ratios of 1:0, 1:1, 1:2.5, 1:5, 1:10, 1:15, 1:40, and 0:1. All mixtures were replicated three times. The soil mixtures were placed in the microplots that had been recently fumigated to eliminate residual *P. tinctorius* inoculum. For comparison with overwintered inoculum, fumigated soil in three microplots was infested with fresh mycelial inoculum of *P. tinctorius* as before. Loblolly pine seeds were planted and the microplots were mulched with fumigated pine straw. Seedlings were maintained as in previous tests. After 8 mo, all seedlings were lifted and the degree of ectomycorrhizal development by *P. tinctorius* was determined. Representative ectomycorrhizae were surface sterilized and placed on MMN medium to reisolate *P. tinctorius*.

Results

Monthly root examinations revealed that the mycelial inoculum of *P. tinctorius* was effective in forming ectomycorrhizae on seedlings as early as May, one month after

TABLE 1. Average soil temperatures (°C) during July through August 1972 as influenced by soil mulches under loblolly pine seedlings (5 and 10 cm depths averaged).[1]

Time	Pine straw	Black coal wastes	Black plastic	Clear plastic	Air
0600–0800	21.0	20.7	20.5	20.5	20.1
0800–1000	24.7	24.5	24.7	24.8	23.8
1000–1200	26.9	28.8	30.1	30.1	29.0
1200–1400	28.0	31.1	32.4	33.3	30.1
1400–1600	29.1	33.2	34.4	34.8	31.7
1600–1800	29.7	34.9	35.6	37.4	30.4
1800–2000	27.4	28.2	27.9	28.2	23.8
2000–2400	20.9	21.1	21.3	20.8	20.4
Range	20–38	19–45	19–46	19–47	20–37

[1] Averages of 50 to 90 measurements per 2 hr interval between 0800 and 1800, 14 measurements per interval between 1800 and 0800.

seedling emergence. Concurrent with this was an increase in seedling height growth which persisted throughout the experiment. Ectomycorrhizae were not detected on seedlings in the noninfested soil until late June. Ectomycorrhizal development from basidiospores of *P. tinctorius* was sporadic and first detected in August, about 6 wk after soil infestation. Height growth of these seedlings was similar to that of seedlings in the noninfested soil.

From mid-August to October, basidiocarps of *P. tinctorius* developed on soil outside of all microplots containing mycelial inoculum. A total of 19 basidiocarps, 1 to 4 cm in diameter, were rogued. Basidiocarps appeared first around microplots with pine straw mulch, then around the other microplots 2 to 3 wk later. One basidiocarp formed in late October on soil on the outside of one microplot in the basidiospore plus clear plastic mulch treatment.

Soil temperatures were influenced by type of soil mulch immediately after mulch placement in early July (Table 1). However, by early October the seedlings were large enough to shade and diminish the influence of the mulches. Generally, daytime soil temperatures at 10 cm depth were about 1°C lower than those at 5 cm. At night, all soil temperatures were similar. Clear plastic mulch caused the highest soil temperatures, followed in order by black plastic, coal wastes, and pine straw. Extreme high temperatures were similar under all mulches except pine straw, but the extremes persisted longer (3 hr or more) under the plastic mulches. Diurnal differences of soil temperatures under black coal wastes (23° to 25°C), black plastic (24° to 26°C), and clear plastic (27°C) were much broader than were those recorded under pine straw (15° to 17°C) or those in the air (17°C). Differences in soil temperature, however, did not significantly influence seedling growth or ectomycorrhizal development (Table 2).

Seedlings grown in soil with mycelial inoculum of *P. tinctorius* were significantly larger and weighed twice as much as seedlings grown in soil with basidiospores or in noninfested soil. The entire feeder root systems of seedlings that received mycelial inoculum were colonized by *P. tinctorius* and over 90 percent of the feeder roots were ectomycorrhizal (Fig 1B,C). Other fungal symbionts were not detected on roots of these seedlings. These ectomycorrhizae were yellow-brown and prolifically branched; some had as many as 36 root tips. Numerous yellow-brown hyphal strands radiated from them and permeated the entire soil volume of each microplot. The hyphal strands were easily traced under or through cracks in the wood frames to basidiocarps of *P. tinctorius* outside the microplots. Microscopic examination of these ectomycorrhizae revealed the fungus mantle to be 40 to 86 μm thick and

TABLE 2. Growth of 8-mo-old loblolly pine seedlings in fumigated soil with and without Pisolithus tinctorius *under different soil mulches.*[1]

Soil treatment and measurements		Pine straw	Coal wastes	Black plastic	Clear plastic	Mean of mulches
Infested with vegetative mycelium of *P. tinctorius*						
Height	cm	37.5	35.2	36.5	34.2	35.9
Stem diameter	mm	8.4	8.2	8.2	7.9	8.2
Foliar-stem dry wt.	g	8.9	8.9	9.4	8.2	8.9
Root dry wt.	g	2.6	2.8	2.7	2.4	2.6
Ectomycorrhizae	percent	90.4	93.0	92.2	93.0	92.2
Infested with basidiospores of *P. tinctorius*						
Height	cm	30.9	24.9	24.8	24.9	26.4
Stem diameter	mm	6.9	6.0	6.0	5.9	6.2
Foliar-stem dry wt.	g	5.6	4.0	4.0	3.7	4.3
Root dry wt.	g	1.5	1.4	1.4	1.3	1.4
Ectomycorrhizae	percent	57.5	61.4	60.8	41.9	55.5
Noninfested (control)						
Height	cm	24.9	25.2	23.8	26.6	25.1
Stem diameter	mm	5.6	5.9	5.6	6.2	5.8
Foliar-stem dry wt.	g	3.8	4.1	3.5	4.4	4.0
Root dry wt.	g	1.3	1.2	1.1	1.4	1.3
Ectomycorrhizae	percent	44.5	45.0	46.3	44.9	45.4

[1] All measurements of seedlings grown with vegetative mycelial inoculum of *P. tinctorius* were significantly ($P = .01$) larger than those of seedlings from either the basidiospore or noninfested treatments. Seedlings in the latter two treatments were not significantly different.

the Hartig-net penetrated to the endodermis. *P. tinctorius* was reisolated from ectomycorrhizae from each microplot with a frequency between 8 and 76 percent of attempts. Reisolation frequency was not related to type of soil mulch. Vegetative cultures obtained from ectomycorrhizae were similar on MMN agar medium to the isolate of *P. tinctorius* originally introduced. Identification of *P. tinctorius* in these ectomycorrhizae was also confirmed with the FA technique. Other fungal symbionts were not detected on roots of these seedlings.

Seedlings from the microplots inoculated with basidiospores had approximately 55 percent of the feeder roots ectomycorrhizal but only about half of these ectomycorrhizae were formed by *P. tinctorius*. Ectomycorrhizae formed by this fungus were macro- and microscopically identical to those described previously. *P. tinctorius* was isolated from ectomycorrhizae in this treatment with a frequency of 16 to 65 percent per microplot. All ectomycorrhizae visually identified as those formed by *P. tinctorius* or *T. terrestris* were confirmed with the FA technique. Growth of seedlings in this treatment was not significantly different from that of seedlings in the noninfested soil (Table 2). Ectomycorrhizae not formed by *P. tinctorius* were formed by *T. terrestris* and by an unidentified white fungus. The latter fungus formed pure white ectomycorrhizae. *T. terrestris* ectomycorrhizae, present on seedlings in all microplots of this treatment, were primarily monopodial or dichotomously branched (Fig. 1B) and cream-brown in color. The fungus mantles of these typical *T. terrestris* ectomycorrhizae were 12 to 20 μ thick and the Hartig-net was well developed and penetrated to the endodermis. The white ectomycorrhizae were present on several

seedlings in three of the 12 microplots of this treatment. These ectomycorrhizae had mantles 36 to 58 μm thick and the Hartig-net was developed to the endodermis. Neither *T. terrestris* nor the unidentified fungus were reisolated from over 400 surface-sterilized ectomycorrhizae of each type. The unidentified fungus did not react positively to the FA developed for *P. tinctorius* or *T. terrestris*.

About 45 percent of feeder roots of control seedlings were ectomycorrhizal, with *T. terrestris* (confirmed by FA) forming the majority. One or two seedlings in each of five microplots had a few (5 to 10 percent) ectomycorrhizae formed by *P. tinctorius* (confirmed by FA). The unidentified fungus described previously formed white ectomycorrhizae on several seedlings in two control microplots. *P. tinctorius* was isolated with a frequency of 8 to 32 percent from ectomycorrhizae from the five microplots in which it was observed. *T. terrestris* and the unidentified fungus were not reisolated from over 600 surface-sterilized ectomycorrhizae of each type.

Significant differences were not detected in chemical analysis of needles from seedlings or from soil in the different treatments. Needles from all seedlings averaged 2.3, 0.26, 0.93, 0.32, and 0.07 percent extractable N, P, K, Ca, and Mg, respectively. Soil samples averaged 0.056 percent total N, 1.5 percent organic matter, and 36, 75, 325, and 72 μg per liter of extractable P, K, Ca, and Mg, respectively. Soil reaction averaged pH 5.2.

Thirty-eight percent of the seedlings in this study had rust galls caused by *C. fusiforme*. Length of galls was between 1 and 9 cm (mean of 3.3 cm). The soil treatments had no apparent effect on incidence of rust galls, although the largest galls were on the larger seedlings from the vegetative mycelial treatment of *P. tinctorius*. Comparison of the mean heights of 1538 seedlings with rust galls and 432 rust-free seedlings used for measurements revealed that rust infection reduced height growth by over 20 percent.

P. tinctorius survived the winter in soil in the absence of host plants and formed ectomycorrhizae on seedlings in infested: noninfested soil mixtures as great as 1:10 the following year. Nearly all the seedlings grown in nondiluted, infested soil (1:0 mixture) formed ectomycorrhizae with *P. tinctorius* on approximately 75 percent of short roots. In the 1:1, 1:2.5, 1:5, and 1:10 mixtures 60, 35, 25, and 15 percent of short roots were ectomycorrhizal with this fungus. Ectomycorrhizae of *P. tinctorius* were infrequent on seedlings grown in the greater dilutions of infested soil. Over 90 percent of short roots of seedlings grown in soil infested with fresh inoculum had *P. tinctorius* ectomycorrhizae. *P. tinctorius* was reisolated from representative ectomycorrhizae in all soil mixtures.

Discussion

Results show that *Pisolithus tinctorius* can be artificially introduced into fumigated soil, successfully compete with other species of naturally occurring ectomycorrhizal fungi, colonize roots of loblolly pine seedlings, and develop prolific ectomycorrhizae at soil temperatures ranging from 19° to 47°C. Correlated with this prolific development of ectomycorrhizae was a highly significant increase in seedling growth compared to seedlings ectomycorrhizal with other fungal symbionts. The early and rapid colonization of roots by *P. tinctorius* from mycelial inoculum apparently precluded colonization of roots by other ectomycorrhizal fungi, including *T. terrestris*. Root colonization continued at a rate comparable to new root growth, regardless of different soil temperatures created by the soil mulches. This minimized the number of nonmycorrhizal roots available to other fungal symbionts.

The basidiospore inoculum of *P. tinctorius* did not dominate root systems as did the vegetative mycelium. Perhaps there were insufficient viable basidiospores in the suspension, or, more likely, the basidiospores were added to the soil after *T. terrestris* and the unidentified fungal symbionts were already established on seedling roots. Apparently, the ectomycorrhizae that did form from basidiospores of *P. tinctorius* developed on newly formed non-

mycorrhizal roots without replacing other symboints in previously formed ectomycorrhizae. Since *P. tinctorius* from basidiospore inoculum did not significantly replace other symbionts at high temperatures, it seems possible that the replacement of *T. terrestris* by *P. tinctorius* on tree roots on adverse sites may be caused by a combination of high temperature and high concentrations of sulfur, aluminum, and manganese influencing feeder root development and growth.

Production of basidiocarps by *P. tinctorius* indicates that this fungus may, after successful introduction, become widespread in the nursery by wind-blown basidiospores. These spores could also be harvested and used as inoculum to infest fumigated soil in the nursery the following year.

Mycelium in the vermiculite-peat moss substrate appears to be an excellent form of inoculum for *P. tinctorius*. Previously, this type of inoculum of *Pisolithus* was used successfully for ectomycorrhizal synthesis in a special growth room (Marx and Bryan 1970). As discussed by Mikola (1973), other workers have used similar types of inocula of various ectomycorrhizal fungi for mycorrhizal synthesis on tree seedlings.

Examination of particles of vermiculite-mycelium inoculum from soil suggested that mycelium of *P. tinctorius* in the laminated structure of particles is protected from microbial antagonists and other soil factors. The mycelium grows rapidly from inside the vermiculite particle following stimulation or particle penetration by feeder roots. Preliminary tests with grain spawn (Park 1971) permeated with mycelium of *P. tinctorius* showed grain inoculum is rapidly colonized by microbial competitors and is less effective for ectomycorrhizal synthesis.

The use of basidiospores is, undoubtedly, the most practical way to introduce *P. tinctorius*. Theodorou (1971) and Theodorou and Bowen (1973) reported that basidiospores of *Rhizopogon luteolus* coated onto seed of *Pinus radiata* before planting served as functional inoculum for ectomycorrhizal development. Basidiospores are practical since they do not require a growth phase under aseptic conditions and, in the case of *Pisolithus*, they are available in large quantities in nature on certain adverse sites. Furthermore, they do not create logistical problems of weight and bulk as encountered with vegetative mycelium. Variables including numbers per volume of soil, methods of storage, survival in soil, and factors affecting germination must be evaluated, however. A major limiting factor is that basidiospores of *Pisolithus* will not germinate consistently under laboratory conditions; Lamb and Richards (1974) reported that under the best test conditions only 0.38 percent germinated. These authors tested different conditions of pH, temperature, and relative humidity. Apparently synthesis of ectomycorrhizae under controlled conditions is the most reliable means to determine viability of the spores.

The fact that *P. tinctorius* overwintered in artificially infested soil in the absence of seedlings and was subsequently diluted successfully with fumigated soil the following spring has practical implications. Nursery beds could be artificially infested in the spring and a seedling crop produced. After seedling removal and overwintering of infested soil, more nursery beds could be infested the next spring with the overwintered soil containing *P. tinctorius*. This would economically and rapidly expand soil infestation in nurseries.

Loblolly pine seedlings with ectomycorrhizae formed by *P. tinctorius* and by other naturally occurring fungal symbionts reported in this study have been outplanted on different coal spoils in Virginia and on kaolin spoils in Georgia. Results from these outplantings will help determine the feasibility of using ecologically adapted, ectomycorrhizal fungi, such as *P. tinctorius*, to tailor-make tree seedlings for specific or adverse sites.

Literature Cited

HACSKAYLO, E. 1972. Mycorrhizae: The ultimate in reciprocal parasitism. BioScience 22: 577–583.

HILE, NANCY, and J. F. HENNEN. 1969. *In vitro* culture of *Pisolithus tinctorius* mycelium. Mycologia 61:195–198.

LAMB, R. J., and B. N. RICHARDS. 1974. Survival potential of sexual and asexual spores of ectomycorrhizal fungi. Trans Br Mycol Soc 62:181–191.

MARX, D. H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153–163.

MARX, D. H., and W. C. BRYAN. 1970. Pure culture synthesis of ectomycorrhizae by *Thelephora terrestris* and *Pisolithus tinctorius* on different conifer hosts. Can J Bot 48:639–643.

MARX, D. H., and W. C. BRYAN. 1971. Influence of ectomycorrhizae on survival and growth of aseptic seedlings of loblolly pine at high temperature. Forest Sci 17:37–41.

MARX, D. H., and C. B. DAVEY. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. III. Resistance of aseptically formed mycorrhizae to infection by *Phytophthora cinnamomi*. Phytopathology 59:549–558.

MARX, D. H., W. C. BRYAN, and C. B. DAVEY. 1970a. Influence of temperature on aseptic synthesis of ectomycorrhizae by *Thelephora terrestris* and *Pisolithus tinctorius* on loblolly pine. Forest Sci 16:424–431.

MARX, D. H., W. C. BRYAN, and L. F. GRAND. 1970b. Colonization, isolation, and cultural descriptions of *Thelephora terrestris* and other ectomycorrhizal fungi of shortleaf pine seedlings grown in fumigated soil. Can J Bot 48: 207–211.

MIKOLA, P. 1969. Afforestation of treeless areas. Unasylva 23:35–48.

MIKOLA, P. 1973. Application of mycorrhizal symbiosis in forestry practice. p. 383–411, *In* Ectomycorrhizae: Their ecology and physiology, G. C. Marks and T. T. Kozlowski (eds.). Academic Press Inc., New York. 444 p.

PARK, J. Y. 1971. Preparation of mycorrhizal grain spawn and its practical feasibility in artificial inoculation. p. 239–240, *In* Mycorrhizae. E. Hacskaylo (ed.). US Dep Agr Misc Pub 1189, 255 p.

SCHMIDT, E. L., J. A. BIESBROCK, B. B. BOHLOOL, and D. H. MARX. 1974. Study of mycorrhizae by means of fluorescent antibody. Microbiology 20:137–139.

SCHRAMM, J. R. 1966. Plant colonization studies on black wastes from anthracite mining in Pennsylvania. Trans Amer Phil Soc 56. 194 p.

THEODOROU, C. 1971. Introduction of mycorrhizal fungi into soil by spore inoculation of seed. Australian Forest 35:23–26.

THEODOROU, C., and G. D. BOWEN. 1973. Inoculation of seeds and soil with basidiospores of mycorrhizal fungi. Soil Biol Biochem 5:765–771.